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Structure of V-type ATPase from *Clostridium fervidus* by electron microscopy

E.J. Boekema[1], T. Ubbink-Kok[2], J.S. Lolkema[2], A. Brisson[1] & W.N. Konings[2]
Department of Biophysical Chemistry[1] and Department of Microbiology[2], Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, NL-9747 AG Groningen, The Netherlands

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Abstract

F-type and V-type ATPases couple synthesis or hydrolysis of ATP to the translocation of H^+ or Na^+ across biological membranes and have similarities in structure and mechanism. In both types of enzymes three main parts can be distinguished: headpiece, membrane-bound piece and stalk region. We report on structural details of the membrane sector and stalk region, including the stator, of V-type ATPase from *Clostridium fervidus*, as determined by electron microscopy. Besides visualization of the stator structure, one of the main findings is that in certain projections the central stalk connecting V_1 and V_0 makes an angle of about 70° with the membrane. Implications for the subunit arrangement in V-type and F-type ATPase are discussed.

Introduction

F-type and V-type ATPases have similar architectures and it is generally accepted that their catalytic mechanism is the same (Fillingame 1996). In both types of enzymes a headpiece, F_1 or V_1, is connected via a 40–60 Å long stalk to a membrane-bound piece, F_0 or V_0. The headpiece of F-type ATPase consists of 5 types of subunits in a $\alpha_3\beta_3\gamma\delta\epsilon$ stoichiometry with an approx. mass of 400 kDa, whereas the A_3B_3CDE complex of V_1 has a mass of about 500 kDa (Nelson and Taiz 1989). Bacteria have the simplest F_0 structure, consisting of three subunits a, b and c in a ratio of 1:2:9-12. In plant chloroplast F_0 these subunits are named IV for a and III for c. The two b subunits in plant chloroplasts are slightly different and named I and II. The V_0 subunits are generally less well characterized. The c-type subunit in V_0, the proteolipid, has double the mass of the c subunit of F_0, most likely the result of a gene duplication.

Detailed structural information is available on the F-ATPases (Abrahams et al. 1994). The 2.8 Å resolution structure of bovine heart mitochondrial F_1 shows alternating α and β subunits arranged as the segments of an orange ($\alpha_3\beta_3$). The central cavity is occupied with two long α-helices of the γ subunit, which is the major stalk subunit (Abrahams et al. 1994). In the rotary catalysis mechanism the three nucleotide (ATP/ADP) binding sites on the β subunits cycle alternately through the 'open', 'loose' and 'tight' states, according to the binding change mechanism (Boyer 1993). Concomitant changes in the conformation of the β subunits would drive rotation of the central γ subunit in the ATP hydrolysis mode. In support of this model, rotation of γ relative to $\alpha_3\beta_3$ at the time scale of ATP hydrolysis was recently demonstrated experimentally (Sabbert et al. 1996; Noji et al. 1997). In turn, the rotating γ subunit is thought to drive H^+ translocation in F_0 by an unknown mechanism. For the torque exerted by the rotating stalk on F_0 to be transduced in pumping activity, a stator like structure is required that prevents dissipation of energy in a rotation of F_1 relative to F_0 (Junge et al. 1996; Kagawa and Hamamoto 1996).

A complete understanding of the mechanism of the ATPase machinery is not yet possible, because a high-resolution structure of the complete enzyme is lacking. The two small F_1 subunits δ and ϵ could not

be detected in the X-ray density maps (Abrahams et al. 1994), but more recently the δ subunit structure has been determined by NMR (Wilkens et al. 1997) and the ϵ structure by both NMR (Wilkens et al. 1995) and X-ray diffraction (Uhlin et al. 1997). From several crosslinking studies the location of these subunits with respect to the other F_1 subunits could be determined. The ϵ subunit is close to the γ subunit and together they form the stalk connecting F_1 to F_0. The δ subunit binds to the top of the structure, which is surprising because for a long time it was considered to be somewhere in the stalk region, close to ϵ (Lill et al. 1996; Wilkens et al. 1997).

Most of the remaining structural uncertainties concern the F_0 part. The c subunit (or subunit III in chloroplasts) forms a hairpin loop of two transmembrane helices. Based on an NMR high-resolution structure, models for the oligomeric arrangement of this subunit have been constructed. There is an increasing consensus for a dodecameric ring, for structural (Groth and Walker 1997) as well as for functional reasons (Engelbrecht and Junge 1997). This ring of 12 c subunits connects with the stalk formed by γ and ϵ. A total of 4 to 5 c subunits are involved in the interaction with the F_1 stalk (Watts and Capaldi 1997). The arrangement of the position of the a and b subunits relative to the c subunit is the least obvious. The hydrophilic parts of the two membrane-anchored b subunits (or subunit I and II in chloroplast F_0) form a rod-like dimeric structure (Dunn et al. 1992), which binds to δ on the F_1 headpiece (reviewed in Engelbrecht and Junge 1997). The place of the more hydrophobic a subunit is not very clear, but possibly it is located outside the ring of the c subunit oligomer (Singh et al. 1996).

The recent increase in structural data has generated new modelling which outdates many of the older ATPase models where subunits δ, ϵ and b were placed at fancy positions because of lack of precise data. We now can say that there is enough evidence that the structure is likely to be quite similar to models such as proposed by Duncan et al. 1995 and closely to a configuration as presented by Ogilvie et al. (1997) or calculated by Engelbrecht and Junge (1997). The latter models show besides a central stalk a stator comprised of b-subunits as a second connecting mass between F_1 and F_0. The precise arrangement of V_0 subunits is not yet understood in similar detail.

Electron microscopy (EM) is often applied to study low-to-medium detail in complex macromolecular assemblies. In a recent paper we structurally characterized the V-type ATPase of *Clostridium fervidus* by a combination of EM and single particle analysis (Boekema et al. 1997). *C. fervidus* is a thermophilic bacterium which relies completely on Na^+ as the energy coupling ion. The Na^+ gradient across the cytoplasmic membrane is generated by an ATPase that is one out of three V-type ATPases described in the bacterial kingdom to date. The most important finding was the presence of a second stalk besides the central stalk. This additional talk has a length of 130 Å, but a thickness of only about 15 Å. The narrow second stalk is likely to be the stator, necessary for rotatory catalysis. In this paper we give further details of the V-type ATPase structure and its consequences for subunit arrangements in V-type as well as in F-type ATPases.

Materials and methods

The V-ATPase from *Clostridium fervidus* was purified as described by Höner zu Bentrup et al. 1997. Purified protein, stored in 50 mM MOPS buffer pH 7.0, 150 mM KCl, 5mM $MgCl_2$, 5% glycerol, 0.1% Triton X-100, 2mM DTT and 0.1 mg/ml phospholipids (phosphatidylethanolamine and phosphatidylcholine at a ratio of 3:1) was dialyzed for 2 h against buffer without detergent and glycerol and prepared for EM using the droplet method with uranyl acetate as negative stain. Micrographs were recorded with a Philips CM10 electron microscope at 52,000x magnification.

A set of 829 membrane-incorporated V_1V_0 projections, as well as sets of 44 non-incorporated V_1V_0 projections and 330 V_1 projections were extracted from electron micrographs, digitized with a Kodak Eikonix model 1412 CCD camera and analyzed with IMAGIC software (Harauz et al. 1980). Images were band-pass filtered and normalized, and subsequently subjected to multireference alignment, multivariate statistical analysis and classification. In the data set of the membrane-incorporated V_1V_0, a first classification was focussed on the area occupied by the V_1 headpiece plus the stalk region. It revealed 4 clear classes corresponding to two types of views, a trilobed view represented by 240 projections and a bilobed view represented by 400 projections (for definition of bilobed/trilobed views in F_1F_0, see Lücken et al. 1990). The rest of the projections belonged to some fuzzy classes showing no clear detail. Next, projections of the two types were separately processed; in the classifications the mask was focussed only on the stalk area. The two groups of projections of the bilobed

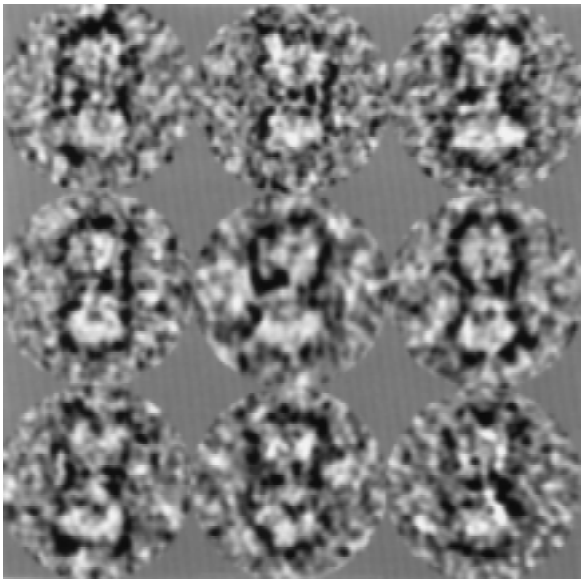

Figure 1. A gallery of images of purified, not incorporated V-ATPase molecules selected from electron micrographs. The images were band-pass filtered and aligned, the first two steps of single particle averaging.

view were asymmetric and showed a difference in handedness. After a mirror translation was imposed on the one half, the two halves were similar at 18 Å resolution and they were further processed together. After classification, the normalized images belonging to the classes were summed without the band-pass filter imposed. In a separately performed classification of 330 projections of dissociated V_1 headpieces no significant differences were found and the best 150 images were summed.

Results

Electron micrographs of V-type ATPase of *Clostridium fervidus* in the presence of small amounts of phospholipids clearly show the tripartite structure of this enzyme: a headpiece is connected via a stalk to the integral membrane sector (Figure 1). In contrast to chloroplast F_1F_0, where the stalk region is relatively short (Boekema et al. 1988a), V-type ATPases show a relatively long stalk (60 Å) and also a larger headpiece (Dschida and Bowman 1992). This makes them interesting objects for investigating fine detail by single particle image analysis. In an analysis of 829 membrane-incorporated V_0V_1 projections they were first classified on features of the V_1 headpiece plus the stalk region. This resulted in a limited number of classes corresponding to a limited number of positions of V_1 on the support film (Figures 2A–D). Two classes show the headpiece projected in a bilobed view, caused by overlap of the six large subunits in two groups (Figures 2A, B). The two views are mirror-related, which is also apparent from two protrusions in the upper half of the headpiece. The stalk is making an angle of about 70° with the membrane. In the other two groups the six large subunits project as three masses, with a symmetrically positioned stalk. In all 4 classes not only a central stalk was visible, but also some vague densities besides the stalk. This prompted us to further separate the data set by a classification on the stalk region.

The classification of the trilobed and bilobed views focussed on the stalk region clearly shows that besides a central stalk a second stalk is visible that connects the V_1 head and V_0 part in the periphery (Figures 2E–H). It has a length of about 130 Å, a thickness of 10–15 Å and widens to ∼30 Å. It runs up to more than halfway the V_1 headpiece and appears either left of right from the central stalk at a distance of about 65 Å, maximally. We have assigned this feature to the stator (Boekema et al. 1997), proposed as a consequence of rotatory catalysis (Junge et al. 1996). Figure 2E gives the impression that the stator attaches the two protrusions seen in the upper left part of the headpiece. In Figure 2F the stator is not seen fully: because the thickening of both the stalk and the stator are closer than in the other views, the stator seems to overlap with the headpiece. In a third class (not shown) the two widenings were even slightly closer. The best two classes of the trilobed view (Figures 2G, H) show the stator on both sides of the projection at a distance as far from the central stalk as in Figure 2E.

Additional structural information was obtained from analysis of single V_1V_0 molecules which were not incorporated in lipid vesicles and present at lower frequency (Figure 1). A classification of 44 of such images again revealed the stator between V_1 and V_0 (Figures 2I, J). The latter image also shows that V_0 is not a symmetrical structure with respect to the stalk: V_0 is wider on the side of the stator. The precise diameter of V_0 can not be determined from such single molecules, because they have a boundary layer of lipid and possibly of remnants of Triton X-100 detergent molecules attached to their hydrophobic surface. But there is a way to estimate the diameter indirectly. V_1V_0 molecules aggregate into small strings and by plotting the number of associated molecules against the total length of the strings the diameter can be es-

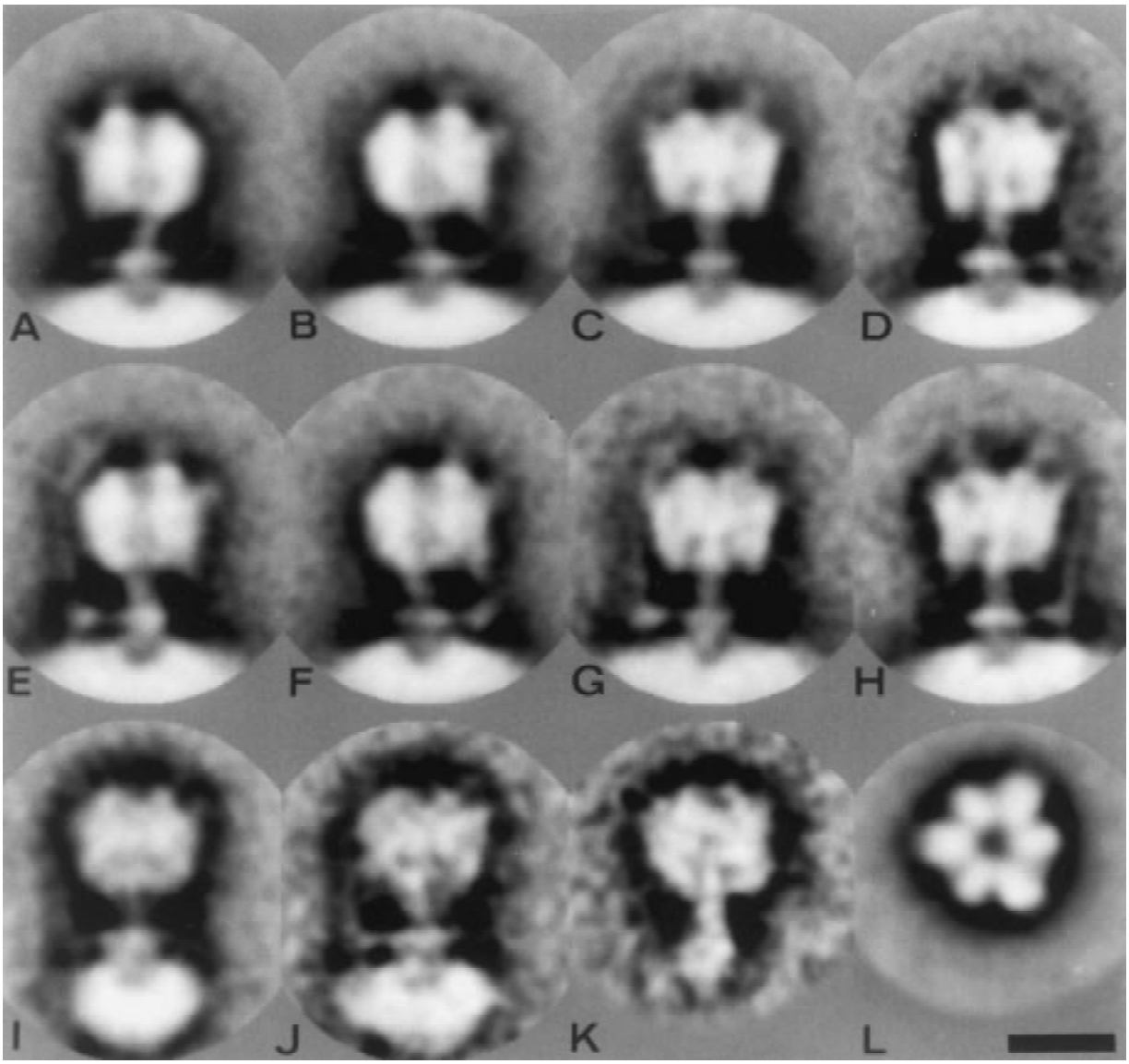

Figure 2. Results from single particle analysis on projections of V_1V_0 and V_1 ATPase. (A–D) classification of membrane-incorporated V_1V_0. Analysis focussing on V_1 plus stalk region resulted in 4 distinct classes with 199, 201, 125 and 115 projections, of which in the images of A–D the better 177, 177, 112 and 47 were summed, respectively. (E, F) two classes (out of three) from a further classification of the projections of A and B with 87 and 89 projections finally summed. (G, H) two main classes (out of six), containing each 42 and 39 projections. (I) total sum of 44 images of non-reconstituted V_1V_0 molecules. (J) class of 12 projections of a classification of 44 images of non-reconstituted V_1V_0. (K) sum of 12 side-views of dissociated V_1 molecules showing a stalk. (L) sum of 150 top-views of dissociated V_1 molecules. The sum, on which no symmetry was imposed, has been rotated in a position in which it would match Figures A and B, if seen from aside. Scale bar is 100 Å.

timated, as shown by Boekema et al. (1988b). From Figure 3 it follows that the diameter of the V_1V_0 molecule is about 80 A. The only problem that remains, however, is the fact that we have determined the diameter of the asymmetrically V_0 molecule in only one direction and do not know if this is the shorter or the longer diameter through V_0 (see 'Discussion').

Leaving the samples for prolonged periods of time at 4 °C resulted in dissection of V_1 from the membranes as evidenced by the appearance of two typical fragments (Figures 2K, L). A limited number of projections show V_1 with the stalk still attached in side-view position (Figure 2K), but most projections correspond to a top view of the headpiece in which

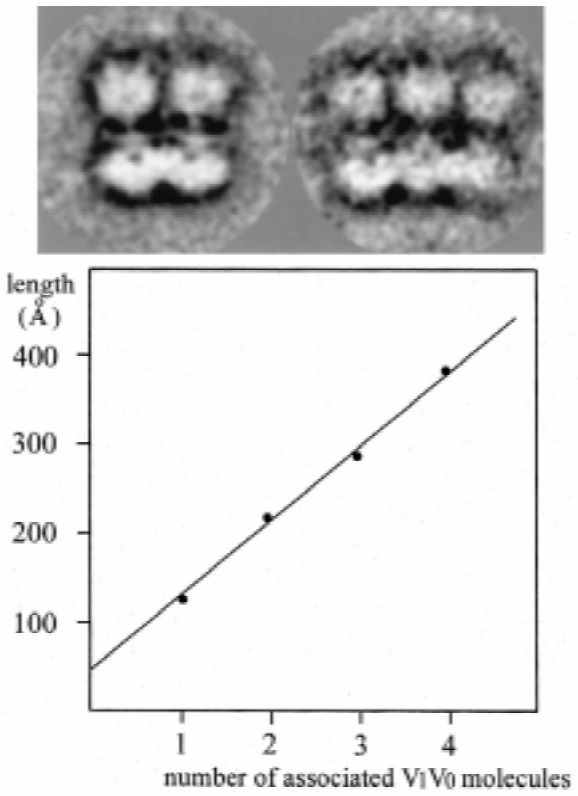

Figure 3. Determination of the diameter of V_0 from 116 strings of 1–4 associated V_1V_0 molecules. The plot of overall length of the strings versus the number of molecules incorporated in the strings follows a straight line. From this we can conclude that in these strings the V_1V_0 molecules are closely packed with lipid (and detergent) molecules merely surrounding the strings as a whole, as previously found for mitochondrial and chloroplast ATPase (Boekema et al. 1988b). From the intersection of the line with the y-axis it follows that the boundary layer around one V_1V_0 molecule is about 48 Å and the diameter of V_1V_0 80 Å. Top panel: sums of 28 and 11 strings of two and three associated V_1V_0 molecules, respectively.

the major V_1 A and B subunits with masses of 66 and 51 kDa (Höner zu Bentrup et al. 1997), respectively, are alternately arranged around an empty center (Figure 2L).

Discussion

The averaged projections determined for V-type ATPase give some hints about the subunit organization of V-type and F-type ATPase. Unfortunately, the *Clostridium fervidus* ATPase subunits are poorly characterized, when compared with F-type subunits from which abundant sequence – and mutagenesis information is available. Nevertheless, there are structural similarities between the individual subunits and we can extract some conclusions from the V-type ATPase projections that are likely to be valid for F-type ATPase.

A first similarity of V-type and F-type is in the headpiece; both have two large subunits present in three copies, resulting in hexagonal types of projections. But the difference in mass between the alternating A and B subunits in V_1 is larger than between α and β in F_1 and this is reflected in Figure 2L, where the difference in mass is obvious. In electronmicrographs of isolated chloroplast F_1 the alternating difference is less striking. Now we can say that here the γ and especially the ϵ subunit tilt the headpiece out of a horizontal plane of the carbon support film causing two neighboring subunits to look smaller in projection (Boekema and Böttcher 1992). Surprisingly, in the V-type ATPase no γ-like feature is visible in the center. Apparently the V-type counterpart of γ is easier lost from the ring of six large subunits, perhaps because its sticks out farther. The V-type ATPase stalk has a length of about 60 Å (Dschida and Bowman 1992), which is significantly longer than the stalk of F-type ATPases (Boekema et al. 1988a; Lücken et al. 1990). This certainly facilitates visualization of the stalk finer details such as the stator (Boekema et al. 1997). In the rather narrow bacterial or chloroplast F_1F_0 stalk region neither the stator structure, nor the thickening of the central stalk have been visualized until now. One reason could be that the averaged particles from cryoelectron microscopy have a more randomlike orientation, which may cause averaging out of such fine details (Lücken et al. 1990).

The position of the central stalk in the images of Figures 2A–D is interesting: although the headpiece stays rather horizontal in projection, the stalk is not exactly vertically situated under V_1 in all positions. It makes an angle of about 70° with the membrane surface. It is clear that such a stalk position could in principle cause large structural rearrangements upon rotation within V_1 (or F_1), necessary for catalytic activity.

Another remarkable feature of the V_0 stalk is its substantially thicker (about 50 Å) base, just above the membrane. This thickening is likely to be a fully water-soluble subunit, rather than a hydrophilic part of a membrane-bound V_0 subunit, since projections showing V_1 with the stalk still attached (Figure 2K) show a similar thickening, excluding the possibility that this is part of a membrane-sector subunit. We do not know which of the V-type subunits comes into ac-

count, but if compared with the F-type ATPase it could be an ϵ-like type of subunit.

An interesting feature of the averaged V_1V_0 side-views is a stain-accumulation just below the stalk (Figures 2E–H). This feature can be best explained as originating from the ring-shaped oligomeric complex of the proteolipid subunit (or subunit c in F-type ATPase). In its isolated form this multimer has a radius of 75 Å (Finbow et al. 1992) and image analysis revealed a central dimple (Finbow et al. 1992). A similar arrangement with dimple is present in F_0 from *Escherichia coli* (Singh et al. 1996). The stain-filled structure observed in the side view projections suggests that the proteolipid multimer is situated symmetrically under the stalk and that the other integral membrane subunits are outside the ring. This conclusion is further supported by image analysis of the non-incorporated V_1V_0 (Figure 2J) which shows an asymmetric overall shape of V_0 under the stalk, with more mass on the left side. Moreover, the stator structure is about 65 Å away from the central stalk and given the fact that the proteolipid oligomer has a diameter of only 75 Å the membrane-anchored part of the stator cannot be inside this structure. The stator of V_0 and F_0 is probably formed by the b subunit, which has a mass between 13 and 17 kDa in V- and F-type ATPase (Supekova et al. 1996). It has an extensive hydrophilic amino acid sequence, predicted to be α-helical and long enough to fit the 130 Å long stator. In prokaryotic F-type ATPases two copies of subunit b are present, which likely form a dimer (Dunn et al. 1992). In V-ATPase the exact subunit stoichiometry remains to be determined. The wide spacing between the two stalks (65 Å), together with the diameter of the proteolipid V_0 oligomer (75 Å) would mean that in the strings of associated V_1V_0 molecules, with a diameter of 80 Å (Figure 3), the repeat is mostly or only formed by the proteolipid oligomers, with the stator part turned away from the string. This would leave about 25 Å membrane-incorporated space for the b- and other subunits. A last remark about V-type ATPase concerns the height of V_0 over the membrane, which is 80–85 Å, (Figures 2I, J and strings), which indicates that in several features the V-type is somewhat larger than the F-type enzyme, where the height is 65 Å and the c-oligomer has a diameter of 62 Å (Boekema et al. 1988b). If the membrane-anchored stator part is as wide as in V_0 (25 Å) and the stator is arranged in a similar way as in V-type ATPase, this would imply that the a-type subunit is arranged between b and c, in contrast to other EM data (Birkenhäger et al. 1995), but in agreement with recent models (Engelbrecht and Junge 1997).

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